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Detection of HPV DNA in Archival Specimens of Cervical Cancer Using In Situ Hybridisation and the Polymerase Chain Reaction

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An archival survey of 98 cervical cancer specimens dating from the 1920s to the 1980s was undertaken to determine whether changes had occurred in the prevalence of human papillomavirus (HPV) DNA. HPV DNA was detected in paraffin sections of cancers fixed in 10% formalin by in situ hybridisation (ISH) using HPV 6, 11, 16, and 18 ^{32}P-labelled DNA probes under conditions of high stringency; and by the polymerase chain reaction (PCR) using 20-mer oligonucleotide primers to amplify 109 bases of the E6 region of HPV 16. In 30 instances results obtained from Southern blot hybridisations which had been carried out on specimens of fresh tissue from the same cancers collected during the 1980s were available for comparison. The rates of HPV DNA detection in cervical cancers ranged from 83% (by Southern or PCR) and 70% (by ISH) on specimens from the 1980s, to 50% and 63% (by ISH and PCR, respectively) on specimens from the 1920s. HPV 16 was by far the most common type, being identified by Southern or ISH in approximately 92% of HPV DNA-positive specimens. No significant change in the prevalence of HPV DNA, or of HPV types, in cervical cancers was found over the 65 year period examined.

KEY WORDS: human papillomavirus, fixation techniques, epidemiology, histological sections

INTRODUCTION

The detection of human papillomaviruses (HPVs) in the cervix has acquired greater significance in the light of investigations which have shown that some of these HPVs are frequently associated with both premalignant and malignant changes. Clinical, epidemiological, and virological surveys suggest that of the twenty or more HPV types found in the anogenital tract, only certain types (especially types 16 and 18, but also types 31, 33, and 35) appear to be associated with malignancy [zur Hausen and Schneider, 1987]. These types have been detected in a high percentage of premalignant cervical lesions, invasive cervical cancers, and in cell lines derived from cervical cancers [Durst et al., 1983; Boshart et al., 1984; McCance et al., 1985; Yee et al., 1985]. In vitro analyses have indicated that the E6 and E7 open reading frames of these HPVs are involved in their transforming activities [Smotkin and Wettstein, 1986; Cornelissen et al., 1990], but other factors—such as smoking or co-infection with other potentially oncogenic viruses—may influence the final outcome [Barton et al., 1988; Iwasaka et al., 1988].

It has been reported that the behaviour of cervical cancer in Australian women appears to be changing, as evidenced by a rise in the prevalence of unusual histological variants and the recent appearance of a more aggressive form of the disease in younger women [Coppleson et al., 1987; Elliott et al., 1989]. To determine whether these changes are associated with variations in the prevalence of HPV DNA, or of HPV DNA types, we undertook an investigation of cervical cancer specimens dating back almost 65 years, using both in situ hybridisation (ISH) and the polymerase chain reaction (PCR) to detect HPV DNA sequences in paraffin sections.

MATERIALS AND METHODS

Specimens

Investigations were performed on a total of 98 histologically diagnosed invasive cervical cancer specimens from patients who had been treated at two major Sydney Hospitals (King George V Hospital and Sydney Hospital) during the period 1922–1987 (Table I). Specimens of fresh cancer tissue collected from 30 women treated during the period 1985–1987 were also available: these had been snap frozen within 30 minutes of their removal from the patient and stored in liquid nitrogen. As we have previously found that fixation in

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formalin-acetic acid-alcohol—used for the fixation of gynaecological specimens at King George V Hospital since 1955—is deleterious to the preservation of HPV DNA by both ISH and PCR [Thompson and Rose, 1991], only specimens which had been fixed in 10% buffered formalin (NBF) were included in the survey. The specimens had been immersed in fixative for between 12–36 hours, then routinely processed and paraffin embedded. The criteria on which the histological diagnoses of cervical carcinoma (or "cervical epithelioma" prior to 1929) had been made were available from the hospital archives' records. For ISH, 5–8 μm sections were cut from the paraffin blocks onto poly-D-lysine coated slides which were stored at 4°C for a maximum of 3 weeks prior to processing; for PCR, 5–8 μm sections were collected into 1.5 ml eppendorf tubes and stored at room temperature.

Southern Blotting

High stringency Southern hybridisation was carried out on the 30 specimens of frozen unfixed cervical tissue using our previously described technique [Zhang et al., 1988]. Briefly, 30–50 mg tissue was finely minced and digested overnight in 600 μl lysis buffer containing 0.5% SDS and 100 μg/ml Proteinase K. The digests were deproteinised by phenol:chloroform:isoamyl alcohol extractions, and the precipitated nucleic acids rehydrated in 50 μl Tris-EDTA buffer pH 8.0. Approximately 5–10 μg extracted biopsy nucleic acids, and 200 pg HPV 6, 11, 16, and 18 DNA standards, were digested with *Bam* H1 or *Pst* I (Pharmacia), then electrophoresed through a 0.7% agarose (Seakem) gel. Nucleic acid fragments were transferred to duplicate GeneScreen Plus (NEN) membranes using the alkaline transfer modification of the method of Southern [Reed and Mann, 1985]. After prehybridisation in 5 × SSC, 0.5% skim milk powder, 1% SDS, 0.6 mg/ml denatured calf thymus DNA for 2 hr, the membranes were incubated overnight at 65°C in prehybridisation solution containing 2–3 × 10^6 cpm/ml denatured HPV probe DNA (purified HPV 6/11 or 16/18 HPV DNA labelled with ^{32}P-dCTP: each mixed probe was used for one of the duplicate membranes). The membranes were washed 3 × 15 min with 2 × SSC/0.1% SDS, and 3 × 15 min in 0.2 × SSC/0.1% SDS at 60°C, then autoradiographed for 3–5 days at −70°C.

In Situ Hybridisation

This was performed using minor modifications of our previously published protocol [Thompson et al., 1990]. Positive controls for HPV 16 (CaSki and SiHa cells) and 18 (HeLa cells) were routinely included: these consisted of cell monolayers which had been fixed in NBF for 20 min. Negative controls were sections of normal neonatal foreskins which had been fixed in NBF for 18 hours. Briefly, the sections were deparaffinised, treated with proteinase K (10–50 μg/ml), washed in 25 mM Tris-HCl, 2 mg/ml glycine buffer, re-fixed in 0.1% glutaraldehyde, acetylated (0.25% acetic anhydride in 100 mM Triethanolamine pH 8.0) for 10 min, and dehydrated. The sections were covered with hybridisation mixture (50% deionised formamide, 5 × SSC, 400 μg/ml calf thymus DNA, 0.02% PVP, 0.02% Ficoll, 1 mg/ml BSA, 40 mM Tris-HCl, approximately 0.1 ng/μl ^{32}P-labelled HPV DNA probe [HPV 6/11, 16, or 18]: final activity of mixture 1-2 × 10^5 cpm/μl). A coverslip was applied and target and probe DNAs were simultaneously denatured (95°C for 8 min). The coverslips were then sealed with rubber cement and the slides placed into moist chambers for incubation at 37°C for 15–18 hours. After high stringency washing (3 × 30 min in 2 × SSC/1 mM EDTA; 2 × 10 min in 0.2 × SSC/1mM EDTA at 60°C; then 30 min in 0.1 × SSC/1 mM EDTA) the slides were dehydrated, coated with Ilford K2 nuclear tract emulsion, dried for 1–2 hr, packed into lightproof boxes, and exposure continued at 4°C for 2–8 days. Following development and fixation (Kodak D19/Ilford Hypam rapid fixer), the slides were washed 15 min in cold tap water, lightly stained with rapid haematoxylin/eosin, washed in 0.1 × and 2 × SSC, then dehydrated and mounted in D.P.X.

Polymerase Chain Reaction

The two oligonucleotide primers for PCR (Fig. 1) were synthesised to correspond with sequences in the E6 region of the HPV 16 genome encoding the E6 and E6* transcripts [Shibata et al., 1988; Cornelissen et al., 1990]. This HPV type was selected for PCR amplification since we have previously found that HPV 16 is by far the most common type currently found in the cervices of Sydney women, particularly those with cervical abnormalities [Zhang et al., 1988; Morris et al., 1990; Law et al., 1991]. Single paraffin sections were deparaffinised using 2 × 400 μl xylene, washed twice with 95% alcohol, and the resulting pellets desiccated and stored at −20°C. Thirty microlitres of reaction

Primer 1 (downstream):

5' ATTAGTGAGTATAGACATTA 3'

Primer 2 (upstream):

5' GGCTTTTGACAGTTAATACA 3'

Fig. 1. 20-mer oligonucleotide primers, corresponding to sequences homologous with the E6 open reading frame of HPV 16, used for the amplification of HPV 16 DNA by PCR.

mixture (67 mM Tris HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 1 mM 2-mercaptoethanol, 6.7 μM EDTA, 0.15% Triton X-100, 200 μg/ml gelatin, 2 mM $MgCl_2$, 250 μM of dNTPs [dATP, dCTP, dTTP and dGTP], 2 μM of each primer, 2 U Taq polymerase [Biotech International]) were added to each tissue pellet. Positive (10–100 ng total nucleic acids extracted from CaSki cells) and negative (reaction mixture only and nucleic acids extracted from neonatal foreskins) control samples were included in each run. After overlaying each reaction mix with 60 μl paraffin, the tubes were heated to 96°C for 15 min, then subjected to 39 cycles of DNA amplification (94°C 30 sec, 54°C 1 min, 72°C 2 min) in a fast thermal sequencer (Bioquest Ltd, Corbett Research). Six microlitres of each amplified product was electrophoresed in 4% Nu Sieve agarose (FML-Bioproducts) in Tris-acetate buffer pH 8.3 at 60 volts for 2 hr. The gels were stained with 1 μg/ml ethidium bromide, then visualised and photographed under UV illumination. Confirmation of the identity of the 109 bp product was carried out by hybridisation of the blotted gel using a ^{32}P end-labelled oligonucleotide E6 HPV probe according to published protocols [Davis et al., 1986].

The possibility of inadvertent contamination of specimens both before and after amplification was minimised by the use of specialised equipment (e.g. positive-displacement micropipettes) and by careful adherence to sample and reagent preparation procedures designed to minimise cross-contamination of samples prior to PCR. A room remote from the sample preparation area and different personnel to those who had prepared the specimens were used for the processing and examination of the amplified products.

Statistical Analyses

The statistical significance of results was determined using the chi-square test with the application of Yates' correction for small numbers. Only P values less than 0.05 ($P < 0.05$) were considered significant.

RESULTS

Analysis of the bands produced by Southern hybridisation performed on the 30 specimens of fresh cervical cancer tissue showed that the overwhelming majority (25/30, 83%) were positive for the four HPV types (6, 11, 16, or 18) used in this investigation (Table I). HPV 16 only was found in 22 specimens; HPV 16 together with HPV 6 was detected in one specimen; HPV 18 only was found in one specimen; and HPV 6 only was found in one specimen. The proportion of cancers containing HPV 16 was very high (77% of the total or 92% of the HPV DNA-positive specimens).

Positive ISH results were indicated by an accumulation of silver grains over the nuclei of cells together with a negligible or low level of non-specific background signal (Fig. 2). The technique produced consistent signals in the CaSki and HeLa control cells (containing an estimated average of 50–100 copies of HPV DNA per cell), but variably weak or absent signals in the SiHa cells (an average of 1–10 copies HPV DNA per cell). The signals seen in the sections of cervical cancers were always focal in distribution—often found in only a few areas of the tumour section—but variable in intensity, ranging from very strong signals which obscured the entire cell to weaker signals which still allowed cell morphology to be readily distinguished. The results achieved using this technique for the detection of HPV DNA are shown in Table I. Overall, HPV DNA was found in 61/98 (62%) of the paraffin blocks examined. Although the detection rates of HPV DNA varied—from 70% in the 1980s specimens to 50% in the 1920s specimens—these variations were not statistically significant ($P > 0.5$). HPV 16 was the commonest type detected, being present in 58% (57/98) of the specimens overall, and 93% (57/61) of the HPV DNA-positive specimens. When compared with the Southern hybridisation results, there was substantial agreement between the two techniques as regards to the presence/absence of HPV DNA and the type of HPV present. However, the less sensitive ISH produced more negative results: HPV DNA being detected in only 70% (as compared with 83% detected by Southern hybridisation) of the 1985–1987 cancer specimens.

Fig. 2. Example of a typical ISH result using ^{32}P-labelled HPV 16 DNA probe on a section from a 1936 cervical cancer.

Successful PCR amplification was evidenced by the appearance of a single band of approximately 109 base pairs (Fig. 3). Only HPV 16 could be detected with the primers used; but nonetheless, very high percentages of cancers tested were positive (75/98, 77%). PCR detected HPV 16 DNA in a further three of the 1985–1987 cancers which had been negative for this HPV type by Southern hybridisation: one of these was the specimen in which only HPV 6 had been identified, while the other two had been specimens in which no HPV sequences had been found by Southern. The rate of PCR positivity ranged from 83% (1980s specimens) to 63% (1920s specimens), but these variations were not statistically significant.

TABLE I. HPV DNA Detection by Southern Hybridisation, In Situ Hybridisation (ISH), and Polymerase Chain Reaction (PCR) in Cervical Cancer Specimens Dating From 1922 to 1987

			HPV DNA				
Years	Technique	N	−ve	6 or 11	16	18	% +ve
1985-87	Southern	30	5	1,1[a]	23[a]	1	83%
1985-87	ISH	30	9	1[a]	20[a]	1	70%
1950-56	ISH	20	8	—	12	—	60%
1940-49	ISH	20	7	1[a]	11[a]	2	65%
1930-39	ISH	20	9	—	10	1	55%
1922-29	ISH	8	4	—	4	—	50%
1985-87	PCR	30	5	ND	25	ND	83%
1950-56	PCR	20	4	ND	16	ND	80%
1940-49	PCR	20	5	ND	15	ND	75%
1930-39	PCR	20	6	ND	14	ND	70%
1922-29	PCR	8	3	ND	5	ND	63%

ND = not determined.
[a]One specimen positive for both HPV 6 and HPV 16.

DISCUSSION

While some published surveys have indicated increases in the prevalence of both cervical HPV infection and of associated pre-malignant (CIN) and malignant changes over the past 25 years [Roberts, 1982; Beral and Booth, 1986; Alasio et al., 1989], other studies employing similar histological and cytological techniques have failed to confirm these findings [Mazur and Cloud, 1984; Armstrong et al., 1986]. However, despite the conflicting speculation on the past epidemiology of cervical HPV infection, most molecular investigations have concentrated on contemporary and prospective studies. To our knowledge, there have only been a relatively small number of published reports describing the results of retrospective surveys for HPV DNA in cervical material [Collins et al., 1988; Rakoczy et al., 1990; Ji et al., 1990]. This situation may be due to a combination of the logistic difficulties in retrieving suitable (e.g., correctly identified) material from hospital archives, together with the technical difficulties inherent in any study of very old material—for example, some of the oldest blocks we located had crumbled away or otherwise deteriorated, and many required re-embedding. Those surveys which have been published have demonstrated a long-standing association between HPV and cervical cancers and premalignancies. One group using ISH to examine a small group (25 specimens) of both CIN lesions and invasive cancers found HPV DNA in lesions dating back to 1932 [Collins et al., 1988]; while the study by Rakoczy et al. of archival Papanicoloaou smears from 1972 to 1987 revealed high prevalences of HPV in both normal and abnormal cervices [Rakoczy et al., 1990]. An ISH survey of 43 invasive cancers removed from Chinese women between 1958–1963 showed that 44% contained HPV DNA [Ji et al., 1990].

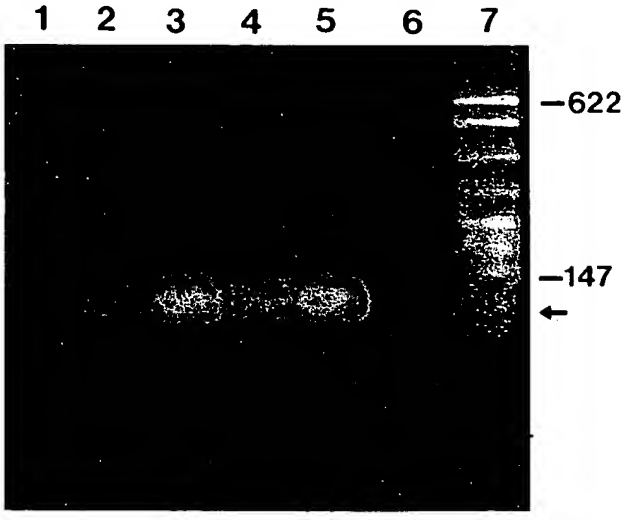

Fig. 3. Result of PCR amplification for HPV 16 DNA in cervical cancer specimens from the 1930s. Positive results are indicated by the appearance of a single band of 109 base pairs (arrow). **Lane 1**, negative control (1 μg foreskin nucleic acids); **lane 2**, positive control (10 ng CaSki nucleic acids); **lanes 3 to 6**, extracts from paraffin sections of cervical cancers (lanes 3, 4, and 5, positive results; lane 6, negative result); **lane 7**: pBR 322-*Msp* I digest size markers.

Although we found some decrease in the HPV DNA positivity rate in cervical cancers over the 65 year period examined, this decline was not statistically significant, and the proportion of HPV 16 DNA detected (as compared with the overall HPV DNA positivity rate) remained constant. In agreement with other investigators from both Australia and other countries [McCance et al., 1985; Collins et al., 1988; Rakoczy et al., 1990; Ji et al., 1990; Grusendorf-Cronen and Cremer, 1990] we found HPV 16 to be by far the most common HPV type detected in cervical cancers. Given the age of many of the specimens we examined it is likely that some decline in DNA detection rates

would be expected—reflecting technical considerations rather than an actual decrease in HPV positivity. The histological fixation and processing techniques in use half a century ago may have adversely affected the preservation of recognisable HPV DNA sequences, even if a technique as sensitive as PCR is utilised for their identification. Various workers have demonstrated that both the type of fixative and the duration of fixation may have a profound effect on the efficacy of specific DNA (including HPV DNA) detection by both ISH and PCR [Moench et al., 1985; Nuovo and Richart, 1989; Ben-Ezra et al., 1991; Thompson and Rose, 1991]. It is possible that such factors may be at least partially responsible for the reported recent twofold increase in the prevalence of HPV detected in formalin-fixed anal squamous cell carcinomas over a 40 year period [Scholefield et al., 1990].

The results which emerged from this survey failed to support the supposition that changes in the prevalence of HPV DNA types may have been responsible for the recent alteration in behaviour of cervical cancer which has been noted in Australian women, manifested by the recent high rates of recurrence and metastasis in young women with early stage disease and no histologically apparent pelvic lymph node involvement [Coppleson et al., 1987; Elliott et al., 1989]. However, it is possible that other, but more subtle, changes in the HPV-host cell relationship may have contributed to the changing character of the disease. We are currently using PCR and other techniques to identify both HPV DNAs and mRNAs in primary tumours in an attempt to clarify whether particular HPV polymorphisms, patterns of genome integration or its expression in the tumour cell are associated with clinical outcome. We are also evaluating the possibility that the identification of HPV nucleic acids in sections of pelvic lymph nodes may be a more sensitive indicator of early pelvic metastases than conventional histologic examination. Such investigations may provide future molecular tools of benefit to the current "staging" techniques for cervical cancer.

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